A comparison of the chemical composition and biological activity of mature and immature honeys: an HPLC/QTOF/MS-based metabolomics approach

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Abstract: Harvesting uncapped immature honey (IMH) followed by dehydration is a typical counterfeit honey production process, but the differences between IMH and capped mature honey (MH) have previously not been well described. In this study, MH and IMH from the *Apis mellifera* colonies in the same rapeseed flower season were compared. MH was found to have lower water content, acidity and higher fructose content. HPLC-Q-TOF/MS based untargeted metabolomic analysis indicated that MH had a distinct metabolite composition to IMH. Targeted metabolomic analysis on 20 major polyphenolic constituents showed higher accumulation in MH. MH had greater bacteriostatic effect and stronger free radical scavenging effect. Whilst both honeys mitigated cell damage caused by H_2O_2, the effective dosage of IMH was higher and its inducing effect on the anti-oxidant gene expression was weaker. Overall, MH was shown to be of better quality than IMH not only because of its richer polyphenolic composition, but also due to its stronger biological activity.

Keywords: honey, mature, immature, metabolomic analysis, bioactivity, HPLC-Q-TOF/MS.

Introduction

Honey is a miraculous product resulting from millions of years of coevolution between plants and honey bees, *Apis* species.[1] It is a natural sweetener that originates from the plant nectar or honey dew collected by bees and further matured inside the bee hive.[2] Bees build a band of honeycomb above the brood cells in their nests to store honey and pollen. The mature honey is capped with white wax for long-term storage. [2] Stored honey and pollens act as food sources, whilst the honeycomb band provides insulation during the winter period or on days without foraging activities.[3]

The process of honey maturation begins with the forager bees taking the nectar or honey dew to the hives.[2] The forager transfers these carbohydrates from their stomach to storer bees.[4] Storer bees normally add their own substances, like enzymes from the hypopharyngeal glands to convert the sucrose into glucose or fructose.[5] The acids from the bees' stomach lowers the pH of the IMH. At the same time, the drying process by their evaporation behavior further decreases the moisture of the honey.[2] The duration of honey maturation varies from one to eleven days depending on-colony size, humidity, climatic conditions and the botanical origins of the nectar.[6] After the honey matures, bees cover the honey with a wax lid as protection and to prevent unwanted fermentation and spoilage.[7]

Due to its great value, honey has been subjected to fraud threat since ancient times. Counterfeit honeys remain a serious threat to the global beekeeping business. Typical frauds may involve diluting honey using a variety of syrups,[8,9] lightening honey color using ion-exchange resins,[4] labeling the honey with fraudulent geographical and/or

botanical origins,[10] artificial feeding of bees during a nectar flow and harvesting the immature (uncapped) honey.[11] The latter fraud type is quite prevalent, since some beekeepers think this can increase the honey harvest. The unmatured honey then undergoes dehydration with vacuum dryers, resulting in most physiochemical features still falling within regulatory.[11] Collecting uncapped honey followed manual dehydration is now regarded as an illicit practice. It is already accepted that water content might be a major difference between mature (capped) and immature (uncapped) honey.[11] However, during the natural transformation of nectar into honey, bees can add specific substances. The chemical composition of honey is complex, not only consisting of sugars and water, but also other constituents, including amino acids, vitamins, minerals and plant polyphenolic acids.[12] These components together endow honey with distinct flavors and biological activities.[13] Nevertheless, it remains to be determined whether these minor substances result in significant differences in chemical compositions and biological activities between mature honey (MH) and immature honey (IMH). To understand these two types of honey better, this study compared the chemical composition and biological activities (anti-oxidative and anti-microbial) of MH and IMH.

Materials and methods

Chemicals and reagents

Methanol (MeOH) and formic acid (FA) were purchased from Fisher Scientific Inc (Pittsburgh, PA, USA). Trolox, ascorbic acid, quercetin, gallic acid and other standards were purchased from Sangon biological engineering co. LTD (Shanghai,

China). Solid-phase extraction (C18) was purchased from Waters scientific Inc. LB Nutrient Agar was purchased from Beijing Aoboxing biotechnology co. LTD. *Escherichia coli, Staphylococcus aureus*, and *Bacillus subtilis* were obtained from Institute of microbiology, Chinese Academy of Sciences, China. The solid phase extraction (SPE) cartridges were obtained from Waters (Milford, Mass, USA). All the cartridges contained 500 mg of C_{18}.

Honey sample collection and physicochemical analysis

Raw honey samples were collected from three *A. mellifera* L. colonies in Sichuan, China, during the flower season from March 1st to March 30th, 2019. Three colonies with the same potential were selected from the experimental bee hive. The honey in the colonies was cleared and only a small amount was left for bees to maintain a basic life. The honey collected by bees and brought back to the nest for no more than 24 h was recorded as immature (uncapped) honey (IMH), and the honey stored in the honeycomb with a beeswax seal until the sealed area of beeswax was greater than 70% was recorded as capped mature (capped) honey (MH). Three IMH and MH samples~~,~~ were separately collected from each colony. A total of 18 samples were collected, including 9 MH and 9 IMH, and stored at −20 °C in the dark prior to use.

These 18-batches of rapeseed honey were subjected to chemical analysis. Indicators including water, glucose, fructose, sucrose, acidity and 5-hydroxymethylfurfural (HMF) were determined as previously described.[14]

Preparation of active substances

Five grams of honey sample was added into 10 mL deionized water followed by

sonicating–at 60 kHz for 10 min and centrifugation at 8000 r/min for 5 min. The supernatant was collected and added to the SPE cartridges that were preconditioned initially with 5 mL of methanol (MeOH) and then 5mL of water. The supernatant samples passed through the cartridges at a flow rate of approximately 1 mL/min. The analytes were eluted with 8 mL of methanol. The resulting eluate was dried using a nitrogen stream to obtain immature honey extract (IMHE) and mature honey extract (MHE). Both extracts were stored at -20 ℃.

HPLC-Q-TOF/MS analysis of honey extract

The honey extracts were re-dissolved to a pre-determined concentration with MeOH. The solution was then filtered with a 0.22 μm nylon membrane and placed in a brown vial. High performance liquid chromatography combined with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF/MS, 6545) system was used to perform the chromatographic analysis in the negative ionization mode. An Agilent Zorbax Poroshell EC-C18 column (2.1 mm x 100 mm, 2.7 μm) was used to separate the extracted compounds. Analytes were separated by linear gradient elution with ultrapure water containing 0.1% formic acid (v/v) (A) and MeOH (B) at a flow rate of 0.25 mL min^{-1}. The linear gradient elution program was: 0–1 min, 5% B; 1–6 min, 55% B; 6–20 min, 95% B; 20–26 min, 95% B; 26–27 min, 5% B. The column temperature was set to 30 °C with an injection volume of 2 μL. The parameters of ESI source were as follows: a nebulizer pressure of 40 psi, a capillary voltage of 3500 V, a fragmentor voltage of 120 V, a drying gas (N_2) flow rate of 8 L/min, a drying-gas temperature of 320 ℃ and a mass range of m/z 100−1700.

Determination of total phenolic and flavonoid content

The measurement of total polyphenol content in the honey extracts was determined by the Folinol- Ciocalteu method. 100 µL of the extract was mixed with 100 µL of Folin and Ciocalteu's phenol reagent. The mixture was incubated in the dark for 5 min, followed by the addition of 300 µL sodium carbonate solution (2% w/v) and mixed. The reaction proceeded in the dark for 120 min. The absorbance was measured at 765 nm. Gallic acid was used to calculate the standard curve and the results were expressed as mg of gallic acid equivalents (GAEs) per g of honey extraction.

For the measurement of total flavonoid content, 150 µL of the sample was mixed with 10 µL aluminium nitrate (100 g/L), 10 µL potassium acetate (9.8 g/L) and 330 µL of distilled water. The reaction proceeded in the dark for 120 min. The absorbance of the product was determined at 415 nm. Quercetin was used to calculate the standard curve and the results were expressed as mg of Quercetin equivalents (QEs) per g of honey extraction.

Antioxidant activity

Free radical scavenging ability

Various concentrations of honey phenolic extracts (0.2 mL) were mixed with 0.2 mL of ethanolic solution containing DPPH radicals. The mixture was shaken vigorously and left to stand for 30 min in the dark or until stable absorption values were obtained. The reduction of the DPPH radicals was determined by measuring the absorption at 517 nm. The concentration of the extract providing 50% of radical scavenging activity (IC50) was determined by a linear curve established by mass concentration and

clearance. The results were expressed as mg of Trolox per g of honey extraction. Vitamin C was used as the positive control.

Various concentrations of honey phenolic extracts (0.15 mL) were mixed with 0.25 mL of ethanol solution containing $ABTS^+$ working liquid. The mixture was shaken vigorously and left to stand for 10 min in the dark until stable absorption values were obtained. The reduction of the $ABTS^+$ radical was determined by measuring the absorption at 734 nm. The concentration of the extract providing 50% of radicals scavenging activity (IC50) was determined by a linear curve established by mass concentration and clearance. The results were expressed as mg of Trolox per g of honey extraction. Vitamin C was used as positive control.

Reducing ability

Various concentrations of the honey extracts (0.3 mg) were mixed evenly with 75 μL of sodium phosphate buffer (pH 6.6) and 75 μL of 1% potassium ferricyanide (w/v). The mixture was then incubated at 50 ℃ for 20 min. After 75 μL of 10% (v/v) trichloroacetic acid was added, the mixture was centrifuged at 2000 rpm for 10 min. The upper layer (300 μL) was mixed with 300 μL of deionized water and 60 μL of 0.1% of ferric chloride (v/v). Then the mixture was shaken, and the absorbance was measured spectrophotometrically at 700 nm. The concentration of the extract providing an absorbance of 0.5 (IC50) was determined by a linear curve established by mass concentration and absorbance. The results were expressed as mg of Trolox per g of honey extraction. Vitamin C was used as the positive control.

Cell culture and cell viability assay

Mouse skin fibrocytes L929 cells were incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin at 37 ℃ in an incubator with 5% CO_2. Cells were then passaged once every 1.5 days. The toxicity of the honey extract and H_2O_2 was determined by using a CCK-8 kit (Dojindo, Japan) following the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 550, CA, USA).

Total RNA isolation and quantification

L929 cells were pretreated with designated concentrations of the honey extract for 2 h, then stimulated with 500 μM H_2O_2 for 24 h. Total RNA was collected and extracted using the RNA Pure Kit (Carry Helix Biotechnologies Co., Ltd., Beijing, China). The concentration and purity of the RNA measured using the Nano Drop 2000 ultramicro spectrophotometer. RNA was reverse transcribed by PrimeScriptTM RT Master MIX kit (TaKaRa, Dalian, China) and the product stored at -20 ℃.

Quantitative real-time PCR was implemented using Bioer LineGene 9600 system (Hangzhou, China) with the SYBR premix EXTaq (TaKaRa, Dalian, China) according to the two-step reaction method. The gene-specific primers of selected cytokines were listed in Supplemental Table 1. The expression of housekeeping gene GAPDH was used to normalize the expression levels of these target genes, the specificity was confirmed by dissociation curve analysis and gel electrophoresis. And the relative expression levels of target genes were calculated using $2^{-\Delta\Delta Ct}$ method.

Anti-microbial activity

Anti-microbial activity was measured by an agar diffusion method. LB agar was sterilized and cooled to 60 ℃ and 100 μL bacterial solution (10^6 CFU/mL) was added to each 30 mL agar to prepare the bacteria-containing medium. After the plate was set, the sample solution to be tested was evenly added into a sterilized Oxford cup (100 μL/cup). The negative control was deionized water, and the positive control was ampicillin solution (5 g/mL). Plates were incubated at 37 ℃ for 16 h. A Vernier caliper was used to measure the diameter of the zone inhibition (in mm), and the average values were obtained by repeating the test in triplicate. The results were presented as ~~a~~ mean ± SD.

Statistical analyses

General analysis

Data was obtained from at least three independent experiments and shown as the mean ± SD of the indicated replicates. Statistical differences were analyzed using One way ANOVA test followed by Bonferroni post hoc analysis and Student's unpaired *t*-test $P < 0.05$ was accepted as statistically significant.

Untargeted metabolomics statistical analysis

Raw data obtained by HPLC-Q-TOF/MS system was preliminarily processed to provide structured data in an appropriate format for subsequent data analysis. The resulting data was extracted by the Profinder software tool in the MassHunter Qualitive Analysis Software (Agilent Techologies) and converted into CEF files. The list of all possible components, as represented by the full TOF mass spectral data, was created in this way. Each compound was described by mass retention, time, and abundance. Then

data filtering was performed with Mass Profiler Professional (Agilent Technologies) software. Before statistical analysis, filtration of data matrix by sample frequency was also applied. Only substances with a frequency greater than 70% were selected for further analysis. The sample differences were statistically analyzed by using One way ANOVA test followed by Bonferroni post hoc analysis and Student's paired *t*-test (again, $P < 0.05$ was considered significant).

The materials showing significant difference between groups were matched and analyzed by using Traditional Chinese Medicines (TCM) database (Agilent Technologies). Principal-component analysis (PCA) was also used to analyze the difference between samples, and score plots were produced.

Targeted metabolomics statistical analysis

Targeted compound ion chromatogram was extracted by Mass Hunter Qualitative Analysis software (Agilent Technologies) for all samples. We conducted qualitative analysis according to the retention time, molecular weight and mass spectrometry fragment and quantitative analysis through the external standard method. The peak areas were used to construct standard curves with $R^2 \geq 0.99$. A *t*-test of the quantitative results was performed to analyze the difference of phenolic substances in MH and IMH samples.

Results

Physicochemical analysis

To study the difference between mature (capped) honey (MH) and immature (uncapped) honey (IMH), physical and chemical indicators were assessed as shown in

Table 1. The indexes of MH were as follows: moisture content 18.31 ± 1.52%, acidity 13.67 ± 1.88 mL/kg, total sugar content 73.22 ± 2.71%, fructose content 36.40±0.37%. IMH: moisture content 31.20 ± 1.81%, acidity 19.9 ± 0.42 mL/kg, total sugar content 61.11 ± 2.09%, fructose content 30.86 ± 0.64%. Compared with IMH, MH samples had lower water content, lower acidity and a higher fructose content.

Metabolomic profiling

Untargeted study

We enriched the active components in the honey and analyzed their differences in the honey extracts by metabolomics using Agilent MPP software. In the first step, the molecular features (MFs) that were present in all injections were retained for each species. The total number of the molecules were 3,751 from all injections, and significantly reduced to 3,060 after the filtering step. The results from the data analysis are represented by a Venn diagram (Figure. 1. A). The results showed that 2,572 chemicals were detected in MH, and 2,686 substances were detected in IMH, with 2,198 substances in common. Secondly, molecular features were further filtered based on p-values calculated by one-way ANOVA. A p-value cutoff of 0.05 was set as the filtering standard to maintain the MFs which differed significantly. The final filtering step was conducted using fold change (FC) analysis (Figure. 1. B). The value of FC was calculated as the MF abundance ratios between each of the two groups. Only the MFs with FC of 2.0 or higher abundance were picked out. As shown in Figure. 1. B, each grey dot represents a chemical while the red dots highlight those substances that were significantly up-regulated in MH group compared with those in IMH group. Equally,

the blue dots highlight those substances that were significantly down-regulated. The substances without significant difference between the two groups are represented by gray dots. To evaluate the variation between the two honey samples and simplify the data management, PCA was used. The raw data of 3,060 MFs were subjected to PCA algorithm in the MPP software (Figure. 1. C). The 2D PCA shown represents 67.99% of the total variation. The first principle component (PC1) accounted for 60.29% of the total data variability, while the second one accounted for 7.7%. The distribution areas of the two samples are clearly differentiated. IMH is mainly distributed in the positive axis of PC1, while MH is mainly in the negative axis of PC1.

Targeted study

Twenty types of phenolic compounds were qualitatively analyzed by HPLC-Q-TOF/MS (Figure. 2 & Table 2). Further quantitative analysis showed that except for vanillic acid and syringic acid, the concentrations of 3, 4-dihydroxybenzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, 3-O-acetylpinobanksin, quercetin, hesperitin, pinobanksin, naringenin, galangin, luteolin, kaempferol, apigenin, pinocembrin, 3-(3, 4-Dimethoxyphenyl)-2-propenoic acid, chrysin, caffeic acid, and phenethyl ester in MH was significantly higher than that of IMH. Among them, kaempferol, apigenin, pinocembrin, 3-(3, 4-dimethoxyphenyl)-2-propenoic acid, chrysin and caffeic acid phenethyl ester were only detected in MH.

Comparison on the anti-bacterial activity

We measured the anti-bacterial activity of honey solution against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Table 3). The result revealed that the zones

of inhibition of MH and IMH on *Escherichia coli* and *Staphylococcus aureus* were 19.47±0.31 mm, 14.13±0.68 mm and 17.29±0.78 mm, 12.80±0.98 mm, respectively. However, neither of them showed any obvious inhibitory effect on *Bacillus subtilis* as no zones were observed. The zones of inhibition were significantly higher for the MH than for the IMH (both $P<0.05$), indicating that MH has a stronger bacteriostatic effect than IMH.

Comparisons of the anti-oxidant activities

The experimental results (Table 4) of oxidation resistance showed the content of total phenolics and total flavonoids in the extracts of honey. The MH and IMH samples contained total phenolics of 12.99 ± 0.19 mg gallic acid equivalent (GAE) per gram and 12.20 ± 0.16 mg gallic acid equivalent (GAE) per gram, respectively, and total flavonoids as 3.53± 0.07 mg quercetin equivalent (QE) per gram and 3.41 ± 0.01 mg quercetin equivalent (QE) per gram, respectively. The reduction capacity was 36.97 ± 0.53 mg Trolox equivalent per gram and 28.41 ± 0.76 mg Trolox equivalent per gram in MH and IMH, respectively. DPPH and $ABTS^{+}$ Free radical scavenging power were 21.89 ± 0.08 mg Trolox equivalent per gram and 19.60 ± 0.36 mg Trolox equivalent per gram, 37.82 ± 0.90 mg Trolox equivalent per gram and 32.30 ± 0.81 mg Trolox equivalent per gram.

Subsequently, cell experiments were conducted to further study the anti-oxidant effect of honey. The results showed that when the concentration of H_2O_2 was 500 μM, the anti-oxidative activity of L929 cells was significantly reduced. However, the honey extract had no toxic effect in the range of test concentrations and honey extract

treatment significantly improved the proliferation activity of cells stimulated by H_2O_2 (Figure. 3). MHE concentration of 400 μg/mL significantly increased the expression of anti-oxidant genes HO-1, TXNRD, GCLM and NQO1 (Figure. 4). IMHE was only effective when the concentration of IMHE was 600 μg/mL, and the expression of anti-oxidation gene NQO1 did not significantly promote the effect.

Discussion

To determine whether immaturity of honey might adversely affect honey quality, we performed a comparative study of the differences between the capped and uncapped honeys from the same botanic source (*Brassia campestris L*). We found notable physicochemical and bioactive differences between immature and mature honeys.

The physicochemical indices, including water, sugar, acidity, and HMF, have been previously investigated.[16] These are the basic indicators to characterize the quality of honey. Studies have shown that the physicochemical parameters of honey can be utilized to distinguish between mature and immature acacia honey.[17]

The water content of honey represents a highly important quality parameter for the its shelf life during storage. The origin of honey, nectars normally, contain more than 50% water but bees will further dehydrate the honey in the comb environment.[18] Therefore, early harvest of the immature honey leads to high moisture content.[19] High water content increases the possibility of honey being fermented during long storage periods.[20] The average water content of our MH samples (18.31 ± 1.52 g per 100 g) were below the required threshold standard of the European Regulations of Quality

(no more than 20 g per 100 g). The average water content of IMH samples was well above this standard (31.20 ± 1.81 g per 100 g) suggesting reduced quality and increased possibility of fermentation.

Increased free acidity is an important indicator of microbial spoilage and freshness of honey. When acidity values are above the standard limits, it indicates sugar fermentation due to the formation of acetic acid by alcohol hydrolysis.[21] Depending on the flower source or geographic area, the free acidity of honey varies. As reported in a previous study, honey's acidity ranges from 9.7 to 29.5 meq/kg.[22] In our study, the free acidity of capped rapeseed honey was 13.67 ± 1.88 meq/kg, whereas for uncapped it one is 19.9 ± 0.42 meq/kg. Both results were below the required standard (less than 50 meq/kg).[23] This shows that, the acidity of honey not only depends on nectar source species but is also affected by maturity.

HMF represents an indicator of honey freshness and authenticity whereas high concentrations of HMF in honey indicates overheating and poor storage conditions or adulteration of the honey with inverted syrup.[24] Honey storage at 35°C causes an increase of HMF that exceeds the allowed limit (40 mg/Kg).[25] However, HMF was not detected in our study samples, which means that the honey samples were fresh, but the immature uncapped honey would need to be further dehydrated including a heating process which may increase HMF levels.

Sugar is the main ingredient in all honeys, with concentrations of up to 80%, and explains why honey is the oldest natural sweetener.[26] The sugar content of honey varies from harvest time, due to the flutter of the wings of the bees or the variance

among the nectars.[27] The most abundant sugars in honey are fructose and glucose, with higher quantities of fructose in the majority of honeys.[28] The percentage of fructose and glucose in our samples ranged from 15.5-49.3% and 18.2–48.0%, respectively. Sucrose was not detected or detected in very low amounts in the honey samples, this is not surprising since sucrose is broken down into glucose, fructose and other monosaccharide by enzymes secreted by bees during honey maturation process. Rapeseed honeys normally contain more glucose than fructose, but in this study, glucose content was found to be at lower concentrations than fructose. This may be due to the different geographical origin and the local climate. The results also suggest that a higher percentage of fructose may be produced as honey is matured in the hive for a long time. Mature honey has been shown to have lower water content, higher fructose content and lower acidity than immature honey, and therefore MH appears to be of better quality.

Recently, metabolic profiling methods have been robustly applied to detect the intrinsic similarities and differences in metabolites within biological samples.[29] In the present study, the Mass Profiler Professional (MPP) software was applied in the analysis of the chromatographic data, which enabled us to compare accurately, comprehensively and quickly the major constituents between MH and IMH samples.[30] This MPP analysis has already been used for screening and development of drugs and food inspection, the results of which have been well-recognized in related fields. The method has been confirmed to be precise, accurate and sensitive enough for untargeted analysis.[30,31] The present study is the first application of MPP technology in determining

honey maturity. We analyzed 18 batches of honey samples collected from three different hives. To ensure comparability each comb analysed contained both immature (uncapped) and mature (capped) honey, during a single rapeseed blossom season. We also performed multivariate statistical analysis for classification, prediction, and characterization of marker compounds. Among them, some metabolites, including organic acids, flavonoids, polyphenols, terpenes and others have been reported from honey.[32,33] We investigated the differences in the metabolite composition in honeys under different maturation conditions. As seen in PCA-score plots (Figure. 1. C), IMH samples separate from MH samples, indicating a large difference between the two groups of samples. A volcano plot representing the filtered data is shown in Figure. 1. B The compounds found at significantly ($P < 0.05$) higher levels in IMH than MH samples were organic acids (benzoic acid, linalool, sinapic acid and ganoderic acid *etc.*) , alcohols, some derivatives of acids (ethyl gallate, levistilide), some glycosides, plant alkaloids , and very small amounts of phenolic compounds such as vanilic acid and eugenic acid. The compounds found at significantly ($P < 0.05$) lower levels were caffeic acid, 3, 4-dihydroxybenzoic acid, chlorogenic acid and common phenolic substances.

To further understand the material differences between the two kinds of honey, we chose to analyze the polyphenols that are major active ingredients in honey. A total of 29 types of flavonoids and phenolic acids were studied of which 20 were detected. These compounds were selected as they were predominately the active constituents in honey as well as propolis. We have previously established accurate quantification methods for these chemicals.[14,34] Average concentrations of these polyphenolic

370 compounds varied greatly among samples (between 0.38 ± 0.01 to 158.09 ± 2.89
371 μg/100 g honey). Six of these compounds were only detected in MH, including
372 kaempferol, apigenin, pinocembrin, 3-(3, 4-dimethoxyphenyl)-2-propenoic acid,
373 chrysin and caffeic acid phenethyl ester. Interestingly, with the exceptions of vanillic
374 acid and syringic acid, the content of the remaining 12 substances in MH were
375 significantly higher than those in IMH. These polyphenols are derived from plants, and
376 are known as the key contributors to the honey's color and taste, as well as its biological
377 activities.[35] Of course, phenols can vary depending on nectar plant, bee species and
378 geographic source. [36,37] It is well documented that plant phenolic metabolites change by
379 the action of bee enzymes in honey. The results from the present study show significant
380 changes, which might be due to interaction with some substances in the beehive, like
381 hydrolysis from glycosides to give rise to aglycones. Nevertheless, we only analyzed
382 honey of unifloral origin and it would be interesting to further compare differences
383 between mature and immature honeys from other nectar sources and over different
384 seasons.

385 The anti-microbial activity of honey is clearly established and honey could provide
386 a potential alternative to antibiotics.[39] The possible underlying mechanism of action
387 relies on the ability of honey to generate hydrogen peroxide by the bee-derived enzyme
388 glucose oxidase.[40,41] However, other factors may also contribute to its antimicrobial
389 activity such as high osmotic pressure, acidic environment, low protein content, high
390 carbon to nitrogen ratio, low redox potential (due to the high level of reducing sugars),
391 and a level of viscosity that limits dissolved oxygen and other chemical

agents/phytochemicals. Another potential contributor is the complex composition of honey, which has more than 181 constituents.[42] These include terpenes, pinocembrin, benzyl alcohol, 3, 5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl-3, 5-dimethoxy-4-hydroxybenzoate (methyl syringate), 2-hydroxy-3-phenylpropionic acid, 2-hydroxybenzoic acid, 3, 4, 5-trimethoxybenzoic acid, and 1, 4-dihydroxybenzene. Consistent with previous studies, we found that honey exhibits a bacteriostatic against several pathogens, such as *Staphylococcus aureus* and *Escherichia coli*. *E. coli* is a Gram-negative bacterium that is pathogenic to human and animals and can cause diarrhea and sepsis in children, travelers, piglets and chickens.[43] *S. aureus* is a representative of gram-positive bacteria and infection can cause serious illness in humans.[44] Our results showed that the zones of inhibition of *E.coli* and *S. aureus* by mature rapeseed honey were bigger than the immature rapeseed honeys, thus demonstrating that MH has a stronger bacterriostatic effect than IMH. Nevertheless, both samples had no obvious inhibitory effect on *Bacillus subtilis*. *B. subtilis* is a multifunctional probiotic and is beneficial for human digestion and absorption. It produces subtilis, polymyxin and other active substances to inhibit intestinal pathogenic bacteria.[45]

Honey works as an abundant source of natural anti-oxidants which play an important role in food preservation and human health.[12] Anti-oxidant substances have different mechanisms, such as reducing the damaging effects of reactive oxygen and reactive nitrogen species, inhibiting the effects of enzymes that produce superoxide anions, promoting metal chelation and free radical chain reaction, and inhibiting the

414 formation of active oxidants.[37] In the present study, three standard spectrophotometric
415 methods are used for comparing the *in vitro* anti-oxidant effects of MH and IMH
416 samples: The DPPH test and $ABTS^{+}$ test for radical scavenging activity and the Ferric
417 reducing ability of plasma (FRAP) method for their reducing power.[15] The main anti-
418 oxidants in honeys are polyphenols, including phenolic acids and flavonoids. According
419 to previous studies, the total phenolic content of honey is uncertain, ranging from 0.205
420 mg GAE/g to 1.877 mg GAE/g honey, while among rapeseed honey, it ranges from
421 0.205 mg GAE/g to 0.311 mg GAE/g honey.[15,40] In this study, we studied the total
422 phenolic content of honey extracts, producing results of 12.99 ± 0.19 and 12.20 ± 0.16
423 mg GAE/g honey extracts for MH and IMH, respectively. Although these values are
424 fall within a certain range with previous literature, our results are significantly higher
425 than previously published data.[46] An explanation for this may be that we extracted the
426 honey before testing it. The content of total phenols in the honey polyphenol extract of
427 mature rapeseed honey (12.99 ± 0.19 mg GAE/g extract) was significantly higher than
428 that of immature rapeseed honey (12.20 ± 0.16 mg GAE/g extract). However, there was
429 no significant difference in the content of total flavonoids. Rapeseed honey from
430 different geographical sources has been shown to possess different anti-oxidant
431 capacities. Piotr Marek Kuś et al.[47] studied the anti-oxidant capacity of 10 kinds of
432 rapeseed honey from 8 regions in Poland, finding that the FRAP level was 1.0-1.8
433 (mmol Fe^{2+}/kg), and the average level was 1.3 ± 0.3. DPPH level was 0.3-0.5 (mmol
434 TEAC/kg), average level was 0.4 ± 0.1 (mmol TEAC/kg). The FRAP and DPPH values
435 of MH and IMH samples in our study were smaller than Piotr Marek Kuś et al reported,

436 but the MH has a stronger anti-oxidant activity than the IMH.

437 Honey has a regulatory effect on cell growth and proliferation, metabolism and
438 anti-oxidant enzymes, and has a protective effect on cell damage caused by adverse
439 stimulation.[11] The mechanisms by which honey influences the biological activity of
440 cells is complex .[49,50] In this study, a cell oxidative stress model was applied in mouse
441 fibroblasts (L929) stimulated by hydrogen peroxide as previously established.[51] Firstly,
442 the concentration of H_2O_2 was determined by toxicity testing, as shown in the figure 3.
443 The reproductivity of the cultured cells can be significantly reduced when treated with
444 500 μM H_2O_2, but the honey extract had no toxic effect on cells in the range of tested
445 concentrations. Then cells were pretreated with honey extract prior to 500 μM H_2O_2
446 treatment. Our results demonstrated that the honey extract could significantly improve
447 the cell growth activity. MH showed a positive effect at the concentration of 400 μg/mL,
448 while for IMH is the required concentration was 600 μg/mL. This suggests that honey
449 can counteract the cell damage caused by oxidative stress, with the effect of mature
450 honey more potent.

451 In the meantime, we examined the expression of antioxidant genes (*HO-1, TXNRD,*
452 *GCLM,* and *NQO1*) in cells. The results showed significantly increased expression of
453 anti-oxidant genes in the MH-pretreated cells. However, the effect of IMH was weak
454 and had no significant effect on *NQO1* gene expression. Heme oxygenase 1 (HO-1),
455 catalyzes the decomposition of heme into a series of anti-oxidant and anti-inflammatory
456 molecules that prevent oxidation;[52] NQO1 catalyzes double electron reduction to
457 reduce oxidative damage;[53] GCLM is a subunit of glutamic acid and cysteine synthase,

458 the most important genes in the cellular anti-oxidant defense mechanism;[54] TXNRD
459 (thioredoxin reductase) is involved in many redox reactions in vivo.[55] These anti-
460 oxidant genes are important regulators of NRF-2 signaling pathway.[56] The NRF-2
461 signaling pathway regulates the transcriptional expression of many proteins with
462 detoxification and anti-oxidant defense functions. Our results suggest that honey may
463 affect the cellular oxidative stress response by affecting the NRF-2 signaling pathway.

464 This study performed analysis of mature and immature honey using untargeted and
465 targeted methods, and determined their anti-bacterial and anti-oxidant activity *in vitro*.
466 The results demonstrated that the harvest of honey before the maturity stage can have
467 profound impacts upon its quality. Our study demonstrated using metabolomics data
468 analysis the possibility to that mature honey and immature honey could be distinguished
469 by the metabolite differences between them by means of metabolomics data analysis.
470 Untargeted substance analysis based on Mass Profiler Professional software explains
471 the difference between the two from a macro perspective. Further in-depth analysis of
472 target substance research indicates that effective and beneficial substances are more
473 abundant in mature honey than in immature honey. This is the first time that
474 metabolomics analysis technology was applied to the study of honey quality. Results
475 from in vitro anti-bacterial and anti-oxidant experiments showed that mature capped
476 honey is more effective in inhibiting proliferation of *E. coli and S. aureus*, and may
477 protect mice skin fibroblast L929 cells from the damage of free radicals by enhancing
478 the expression of anti-oxidant related genes after H_2O_2 stimulation. In conclusion,
479 mature honey has a greater value.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information.

Supplemental Table 1: Sequences of the primers used for qRT-PCR

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673

Figure Captions

Figure 1 Discrimination of mature capped honey and immature uncapped honey (MH represents mature honey; IMH represents immature honey). A: Venn diagram of untargeted analysis of MH and IMH with a filtration of samples frequency (70%). The number in the picture represents the number of species of matter. B: Volcano plot of the honey different metabolites for group MH vs IMH ($P<0.05$). C: PCA scores plot of MH and IMH.

Figure 2 Total ion chromatography of honey extracts with negative scanning mode in HPLC-Q-TOF-MS. Red line represents mature honey (MH); Green line represents immature honey (IMH). The samples for 0-1 min are discarded without mass spectrometry.

Figure 3 Effect of H_2O_2 and honey extracts on L929 cells viability. (A). Cells were pretreated with/without the indicated concentrations of H_2O_2 (300 μM-600 μM) and honey extracts (0 μg/mL-600 μg/mL) for 24 h. (B)(C). Cells were pretreated with/without the different concentrations of MHE/IMHE for 2 h and then stimulated with 500 μM H_2O_2 for 24 h.★ indicates the control group for significance analysis. Each result was expressed as the mean ± SD (n = 3); $*P < 0.05$ versus the control group (★); $**P < 0.01$ versus the control group (★); $***P < 0.001$ versus the control group (★).

Figure 4 Effect of honey extracts on the expression of antioxidant related genes in H_2O_2 stimulated cells. L929 cells were pretreated with or without the indicated concentrations of MHE/IMHE for 2 h and were then stimulated with 500 μM H_2O_2 for 6 h. The relative mRNA expression of *HO-1*(A), *TXNRD* (B), *GCLM* (C) and *NQO1* (D) were

696 determined using qRT-PCR. Each result was shown as the mean ± SD (n = 3). ∗∗P <

697 0.01 versus the untreated group (★), ∗∗∗P < 0.001 versus the untreated group (★).

698

Tables

Table 1 Parameters of Mature and Immature Honey[*].

Parameter	MH	IMH
Frucose%	36.40± 0.37[a]	30.86 ± 0.64[b]
Glucose%	34.49± 2.17[a]	30.14 ± 0.72[a]
Sucrose%	2.33 ± 0.17[a]	1.11 ± 0.73[a]
Water%	18.31 ± 1.52[a]	31.20 ± 1.81[b]
Acidity		
meq/kg	13.67 ± 1.88[a]	19.9 ± 0.42[b]
HMF	ND	

[*] In each column, different letters (a, b) mean significant differences ($p < 0.05$). ND means not detected.

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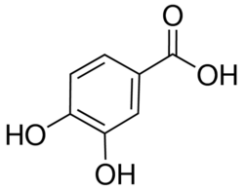
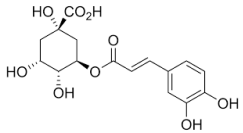
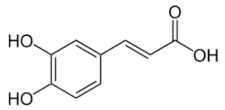
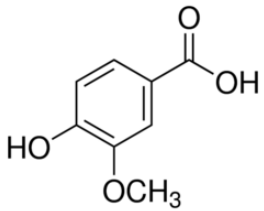
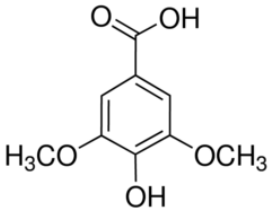
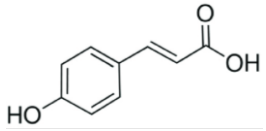
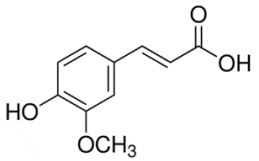
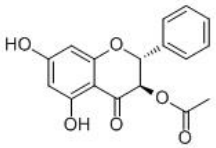
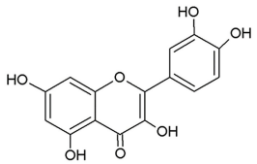
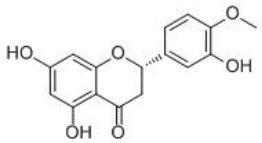
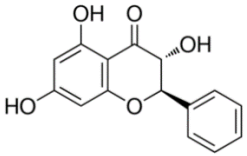
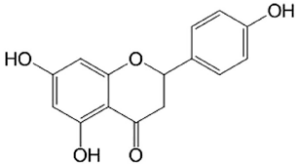
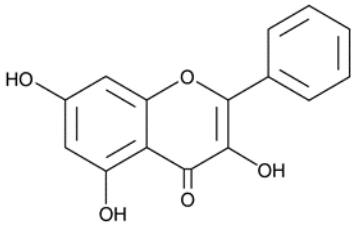
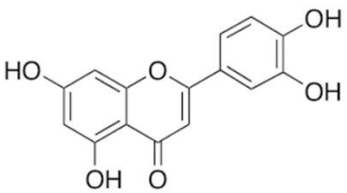
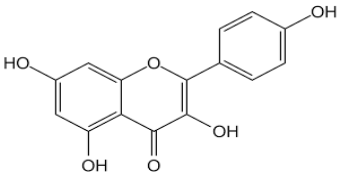
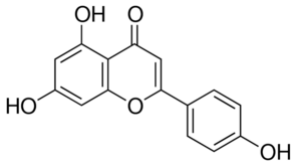
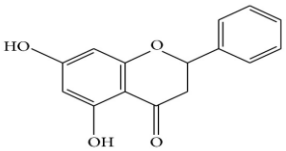
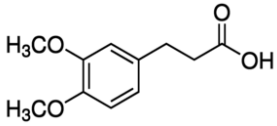
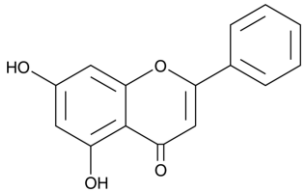
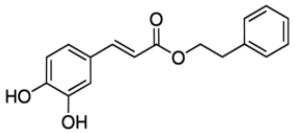
Table 2 HPLC-Q-TOF MS Analysis of Major Phenolic Compounds and Relative Occurrence in MH and IMH*.

Compounds	Molecular formula	Molecular weight	Molecular formulae	[M-H]⁻, *m/z*	RT/min	μg/100gMH	μg/100gIMH	LOD μg/100g	R^2
3,4-Dihydroxybenzoic acid	C7H6O4	154.12		153.0193	4.596	7.20±0.05[a]	3.05±0.05[b]	0.025	0.999
Chlorogenic acid	C16H18O9	354.31		353.0878	5.510	11.70±0.20[a]	4.80±0.09[b]	0.006	0.990
Caffeic acid	C9H8O4	180.16		179.0350	6.035	22.86±0.88[a]	5.37±0.05[b]	0.023	0.992

Vanillic acid	C8H8O4	168.15		167.0350	5.950	51.17±0.58[b]	60.10±0.25[a]	0.250	0.995
Syringic acid	C9H10O5	198.17		197.0455	6.169	6.81±0.04[b]	14.68±0.12[a]	0.147	0.990
p-Coumaric acid	C9H8O3	164.16		163.0401	6.804	9.51±0.52[a]	1.38±0.04[b]	0.013	0.990
Ferulic Acid	C10H10O4	194.18		193.0506	6.950	19.84±1.10[a]	4.36±0.13[b]	0.043	0.998
3-O-Acetylpinobanksin	C17H14O6	314.00		313.0718	11.561	13.53±0.40[a]	0.38±0.01[b]	0.008	0.994

Quercetin	C15H10O7	302.24		301.0354	8.743	134.16±2.41[a]	58.94±0.63[b]	0.057	0.991
Hesperitin	C16H14O6	302.28		301.0718	8.939	158.09±2.89[a]	76.32±0.83[b]	0.012	0.991
Pinobanksin	C15H12O5	272.25		271.0612	8.928	25.74±0.20[a]	0.84±0.01[b]	0.027	0.993
Naringenin	C15 H12 O5	272.25		271.0612	9.098	23.39±0.33[a]	0.88±0.01[b]	0.027	0.993
Galangin	C15H10O5	270.24		269.0455	9.122	8.42±0.24[a]	3.63±0.01[b]	0.018	0.990

Luteolin	C15H10O6	286.24		285.0405	9.952	67.32±1.09[a]	34.82±0.57[b]	0.062	0.990
Kaempferol	C15H10O6	286.24		285.0405	11.391	25.44±0.46	ND	0.078	0.998
Apigenin	C15H10O5	270.24		269.0455	7.792	15.24±0.19	ND	0.039	0.993
Pinocembrin	C15H12O4	256.25		255.0663	12.037	10.95±0.31	ND	0.036	0.992
3-(3,4-Dimethoxyphenyl) -2-propenoic Acid	C11 H12 O4	208.21		207.0663	12.184	19.60±0.23	ND	0.166	0.991

Chrysin	C15H10O4	254.24		253.0506	12.612	18.83±0.97	ND	0.011	0.998
Caffeic acid phenethyl ester	C17H16O4	284.31		283.0976	9.793	12.89±0.26	ND	0.025	0.998

* Detected in negative ionization mode. In each column different letters (a, b) mean significant differences ($p < 0.05$). ND means not detected.

Table 3 Zones of inhibition of MH and IMH*.

	Zones of inhibition (mm)				
	Ampicillin(5μg/mL)	Phenol(10%)	50%MH	50%IMH	Water
E.coli	17.69±0.43	15.40±0.57	19.47^{a}±0.31	17.29^{b}±0.78	--
S.aureus	29.23±0.62	14.40±0.69	14.13^{a}±0.68	12.8^{b}±0.98	--
B.subtilis	0.50±0.08	20.90±0.29	--	--	--

* In each column different letters (a, b) mean significant differences ($p < 0.05$). -- means that there is no observed bacteriostatic zone.

Table 4 Antioxidant Activity of Mature Honey and Immature Honey. Including Radical Scavenging Capacity, Reducing Power, and Total Phenols and Flavone of MH and IMH*.

	FRAP		ABTS		DPPH		Total phenols	Total flavonoids
	IC50 mg/mL	mg Trolox /g	IC50 mg/mL	mgTrolox/g	IC50 mg/mL	mgTrolox/g	mgGAE/g	mgQE/g
MH	1.69±0.02	36.97±0.53[a]	0.86±0.02	37.82±0.90[a]	2.26±0.01	21.89±0.08[a]	12.99±0.19[a]	3.53±0.07[a]
IMH	2.21±0.06	28.41±0.76[b]	1.01±0.06	32.30±0.81[b]	2.53±0.05	19.60±0.36[b]	12.20±0.16[b]	3.41±0.01[a]
Vc (μg/mL)	35.07±0.02		16.76±0.06		28.01±0.02			

* In each column different letters (a, b) mean significant differences ($p < 0.05$). IC50 means the sample concentration providing 0.5 of absorbance was determined by a linear curve established by mass concentration and absorbance. Meanwhile all the results were expressed as equivalent of the corresponding standard reference (mg Trolox equivalation per gram (mg Trolox/g); mg gallic acid equivalent per gram (mg GAE/g); mg quercetin equivalent per gram (mg QE/g)).

Figure graphics

Figure 1

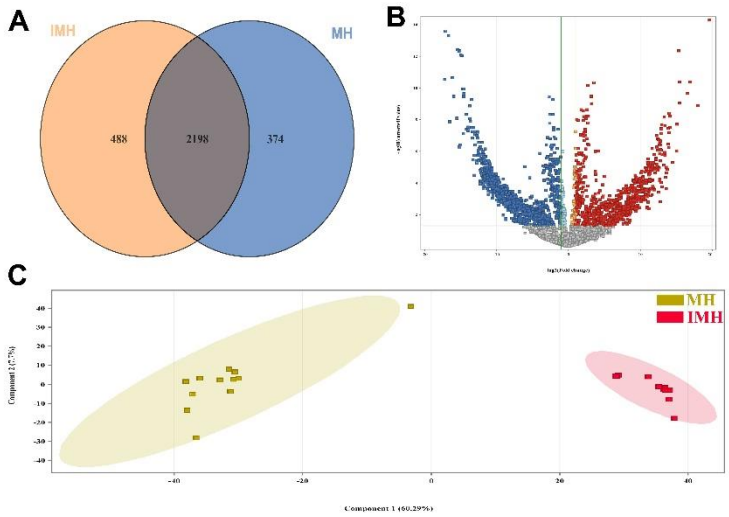


Figure 2

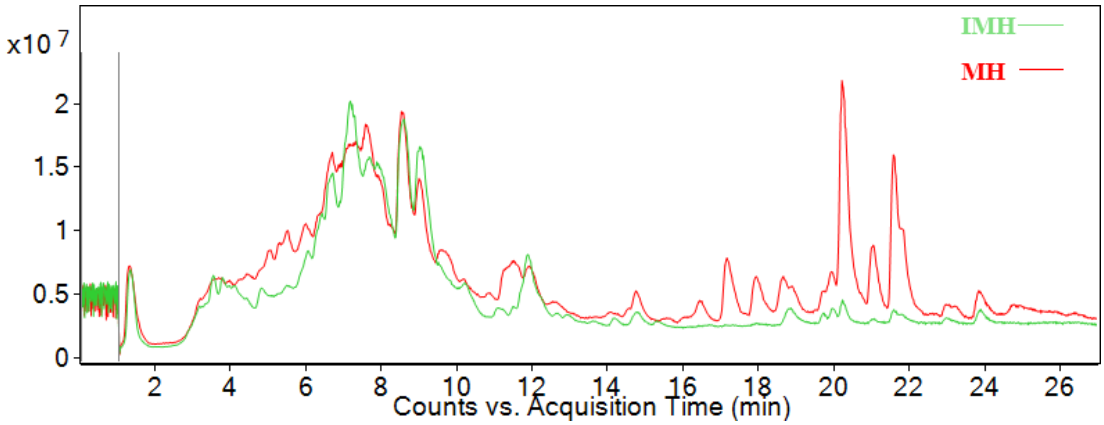

x10 7
2
1.5
1
0.5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Counts vs. Acquisition Time (min)
IMH
MH

Figure 3

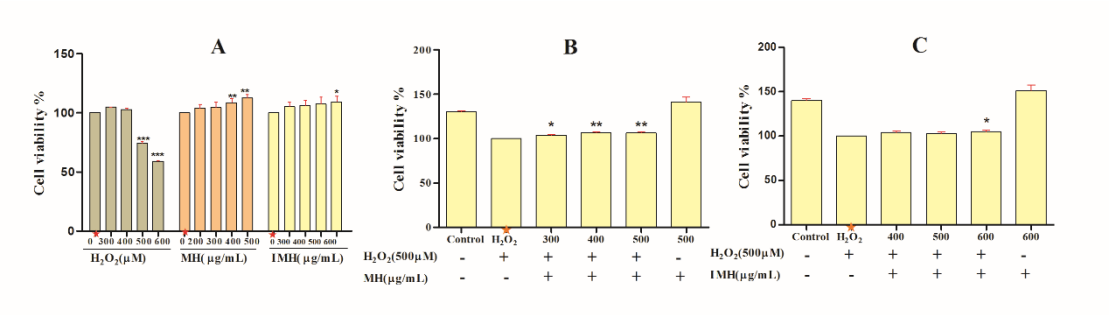
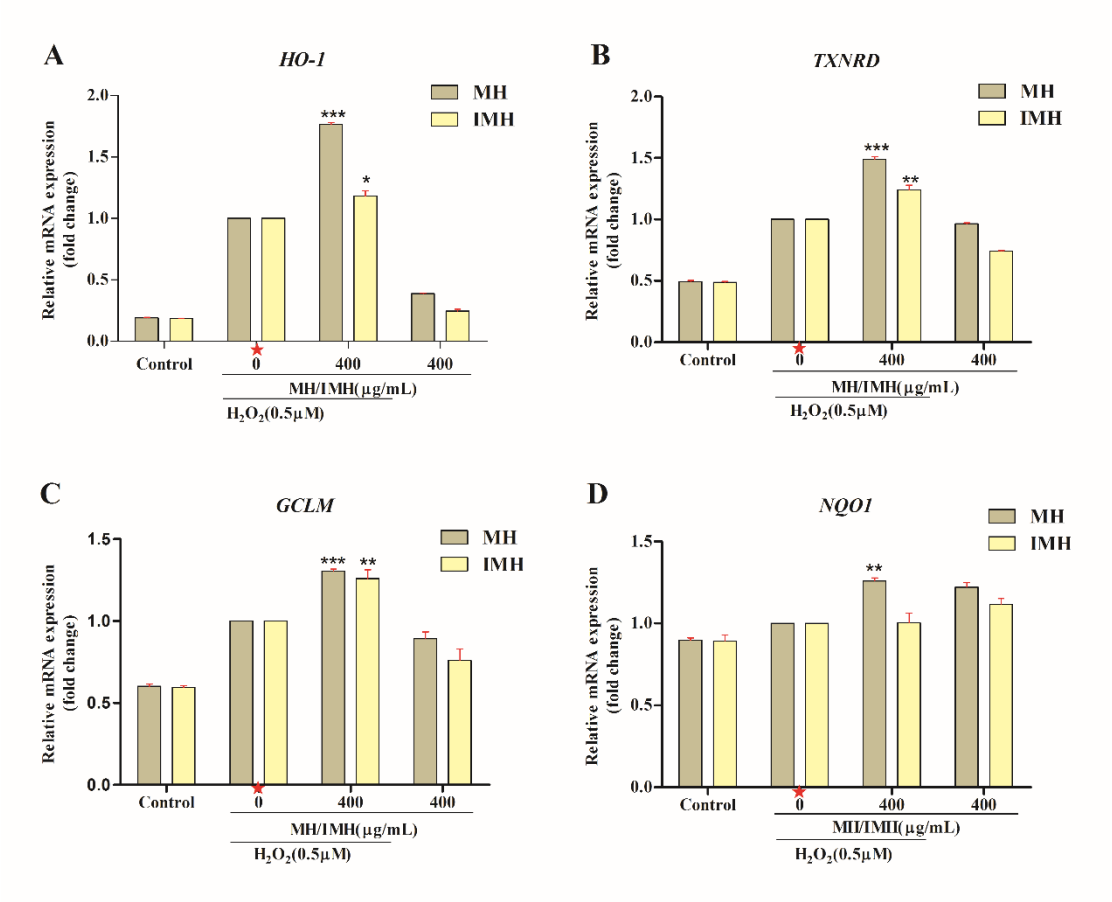

A
B
C
Cell viability %
H_2O_2(μM)
MH(μg/mL)
IMH(μg/mL)
H_2O_2(500μM)
MH(μg/mL)
IMH(μg/mL)
Control

Figure 4


A
HO-1
MH
IMH
Relative mRNA expression (fold change)
2.0
1.5
1.0
0.5
0.0
***
*
Control
0
400
400
MH/IMH(μg/mL)
H2O2(0.5μM)
B
TXNRD
MH
IMH
Relative mRNA expression (fold change)
2.0
1.5
1.0
0.5
0.0
***
**
Control
0
400
400
MH/IMH(μg/mL)
H2O2(0.5μM)
C
GCLM
MH
IMH
Relative mRNA expression (fold change)
1.5
1.0
0.5
0.0
***
**
Control
0
400
400
MH/IMH(μg/mL)
H2O2(0.5μM)
D
NQO1
MH
IMH
Relative mRNA expression (fold change)
1.5
1.0
0.5
0.0
**
Control
0
400
400
MII/IMII(μg/mL)
H2O2(0.5μM)

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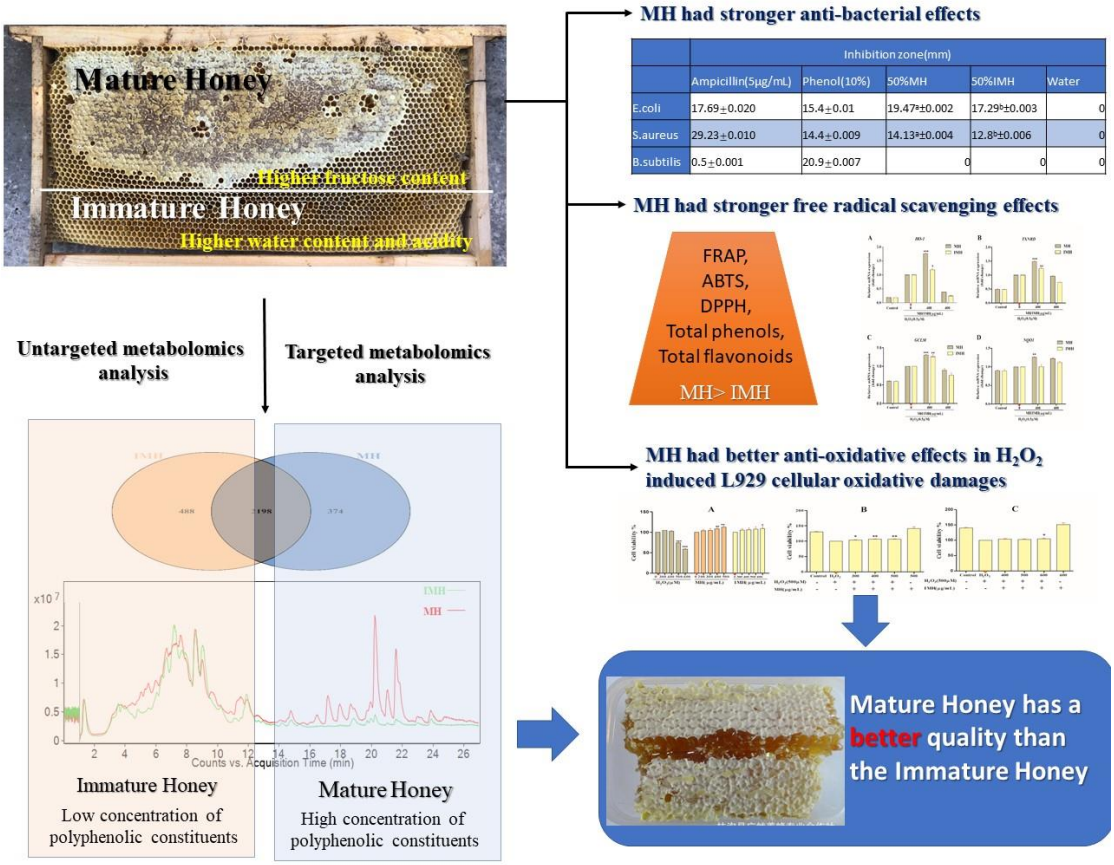

	Inhibition zone(mm)				
	Ampicillin(5μg/mL)	Phenol(10%)	50%MH	50%IMH	Water
E.coli	17.69±0.020	15.4±0.01	19.47ª±0.002	17.29ᵇ±0.003	0
S.aureus	29.23±0.010	14.4±0.009	14.13ª±0.004	12.8ᵇ±0.006	0
B.subtilis	0.5±0.001	20.9±0.007	0	0	0